mRNA DESTABILIZATION: THE ROLE OF THE 3' UTR IN POST-TRANSCRIPTIONAL REGULATION OF WNT8 TRANSCRIPTS

A Senior Scholars Thesis

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ANNIKA DAWN BUTLER

Submitted to the Office of Undergraduate Research
Texas A&M University
in partial fulfillment of the requirements for the designation as

UNDERGRADUATE RESEARCH SCHOLAR

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Associate Dean for Undergraduate Research: Robert C. Webb

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ABSTRACT

mRNA Destabilization: The Role of the 3'UTR in Post-transcriptional Regulation of Wnt8 Transcripts. (April 2010)

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wnt8 is essential for normal embryonic development in vertebrates: severe developmental malformations occur when *wnt8* is either over- or under- expressed. Therefore, *wnt8* has to be activated at a specific time and for a specific duration to promote proper development. While Wnt8 signaling is regulated at several levels, factors that regulate the extent or duration of Wnt8 signaling are not well understood. One possible mechanism is through the action of microRNAs (miRNAs), 21-22 bp oligomers that are complementary to particular mRNA binding sites found in the 3' untranslated regions (3' UTRs) of target transcripts. The binding of miRNAs to specific sites in mRNAs causes one of two fates: the mRNA is degraded or translation of the transcript is suppressed. The zebrafish *wnt8* locus is complex and produces transcripts with alternative 3' UTR elements, each of which has several putative miRNA binding sites. To advance our understanding of *wnt8* regulation, we are testing whether the zebrafish *wnt8* 3'UTR elements destabilize *wnt8* transcripts, and we will pinpoint the UTR

sequences that mediate this function. Our results indicate that the zebtrafish *wnt8* 3' UTRs destabilize transcripts in a similar fashion and in a sequence dependent manner. This mode of regulation is not tissue restricted. By making deletions, it was found that one of these alternate 3' UTRs contains much of its activity within a 5' portion that is 120 nucleotides in length. However, this portion does not recapitulate all of the UTR activity which is indicative of a synergistic effect of multiple regulatory elements such as through miRNAs.

DEDICATION

I am dedicating this thesis to Dr. Arne C. Lekven, a terrific professor and mentor.

It has been a tremendous privilege to participate in the research in your lab. Thank you for all of your guidance, motivational pep-talks, and support over the years. You have allowed me to grow as a scientist and have inspired me in countless ways. Words cannot describe my gratitude.

ACKNOWLEDGMENTS

I would first like to acknowledge my parents, Heide and Larry Butler, and my siblings, Lisa and Collin. Without their love and encouragement, none of this would have been possible.

Thank you to the graduate students in the Lekven lab: Kevin Baker, Holly Gibbs, Anand Narayanan, and Amy Whitener. All of your advice, patience, and laughs made this a terrific environment to learn and develop skills as a researcher.

NOMENCLATURE

EGFP	enhanced green fluorescent protein
hpf	hours post-fertilization
mRFP	red fluorescent protein
QPCR	quantitative polymerase chain reaction
UTR	untranslated region

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CHAPTER I

INTRODUCTION

The main body plan is initially laid down by specifying the 2 main axes: the dorso-ventral axis and the antero-posterior axis. Several genes are involved in this pattern formation and changes in gene activity throughout development are essential in forming proper structures. Intercellular signaling is an important form of communication between cells that promotes proper gene expression at the correct time and place. One such key intercellular signal that is necessary for defining both the antero-posterior and dorso-ventral axis is Wnt.

Wnts are a large family of secreted signaling proteins that play important roles in development and disease by influencing cell differentiation and cell fate at various stages. In the canonical Wnt pathway, Wnt proteins act on cell surface receptors and their binding causes a signal to be transduced across the membrane. The main result of Wnt binding is that β-catenin is not prepared for ubiquitin mediated decay. The stabilized β-catenin can enter the nucleus from the cytoplasm and regulate expression of target genes by interacting with certain transcription factors (Tolwinski and Wieschaus, 2004). This mechanism is utilized in dorso-ventral axis patterning in which β-catenin is stabilized on the dorsal side of the embryo. A signaling center that sets the dorso-ventral polarity is arranged on the dorsal side of the embryo through the actions of β-catenin and other

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This thesis follows the style of Developmental Cell.

factors. Similarly, β-catenin is restricted to the posterior end of a developing embryo to play a role in specifying the antero-posterior axis.

While there are several wnt genes, one of great importance is wnt8. In vertebrate embryos, *wnt8* is expressed in mesoderm progenitor cells during gastrulation and is essential for normal dorsoventral and anteroposterior embryonic axis patterning (Christian and Moon, 1993). Cell fate specification is regulated by *wnt8* signaling to both mesoderm and ectoderm progenitors (Christian and Moon, 1993; Lekven et al., 2001). Remarkable deformities are observed when *wnt8* is either under- or over- expressed. In its absence, the posterior body fails to form properly. *wnt8* mutant embryos lack tails and show brain patterning defects. Conversely, when *wnt8* is over-expressed, resulting embryos lack heads and show exaggerated posterior body formation (Lekven, et al. 2001). These mutants, in which *wnt8* is either over- or under- expressed, do not survive past embryonic stages. In zebrafish, the *wnt8* locus encodes for two protein coding regions that are in tandem. Other vertebrates such as humans have only one Wnt8 ortholog. In the major mRNA that is produced, the transcript is bicistronic and the two protein coding regions are separated by an 800 base pair untranslated region (UTR) that is termed “the linker” and another UTR is found at the 3’ end of the transcript that is 150 bases in length and is termed “the *wnt8*.2 3’UTR. The two proteins that are produced are termed Wnt8.1 and Wnt8.2 and the second arose from gene duplication. While the two proteins are 70% identical, they do have differences in activity.

Gene regulation of *wnt8* is intricate and occurs at several levels. However, in this research project, we are concerned with post-transcriptional regulation in zebrafish, which is attributed to UTRs found downstream of the protein coding region. Two possibilities of regulation are through nonsense-mediated decay (NMD) or through the action of microRNAs (miRNAs). NMD occurs when introns have not been spliced properly out of the mRNA. These introns contain a termination codon, and translation would yield a shorter peptide and an improperly functioning protein. Nucleases are recruited to destroy the improperly spliced mRNA in a process termed nonsense-mediated decay (Hartl and Jones, 2009). Alternatively, miRNAs are 21-22 nucleotide sequences that are associated with a complex of proteins. These sequences are complementary to miRNA binding sites found in the 3'UTR of the mRNA. Binding of these miRNAs can cause one of two fates: either the mRNA is degraded, or translation of the protein coding region is suppressed (Carthew and Sontheimer, 2009). Past research has shown that the *wnt8* 3'UTR in frogs is of importance for transcript stability and mRNA translation levels, but it has not revealed whether this was through a miRNA-dependent mechanism (Tian et al, 1999). We hypothesize that the 3'UTR elements are crucial for regulating the expression, and therefore the activity, of *wnt8* in zebrafish.

CHAPTER II

METHODS

Plasmid construction for making transgenic lines

Plasmids containing the linker region and wnt8.2 3'UTR were constructed. Two plasmids were used as vectors. pT2A6 contains EGFP driven by the wnt8 promoter. The second plasmid, pT2AL200R150G contains EGFP driven by the EF1-α promoter, which drives ubiquitous expression. These two plasmids are tol-2 based vectors that have an ampicillin marker. The inserts were ligated into the ClaI site of each vector which is located between the EGFP protein coding sequence and the SV40 poly-A tail. The vector was prepared by performing a restriction digest with ClaI and a phosphatase reaction was done on the linearized DNA. The inserts of the linker region and wnt8.2 3'UTR were amplified through PCR. Ligation reactions were set up with a Ligase-free control and a 3:1 concentration of insert to vector. Transformation of the plasmids into XL10 Gold competent cells was then carried out and bacterial cells were grown on ampicillin plates. Mini preps to isolate the plasmid of interest were then completed. Because blunt end ligations were performed, the insert ligated in either the forward or reverse direction.

Transgenic lines

Once all 8 plasmids were constructed, they were injected into 1 cell stage zebrafish embryos. The injection solution included transposase. The fish were raised to adulthood, then crossed to wild type zebrafish to screen for EGFP positive embryos. These embryos that were heterozygous for the transgene were raised to maturity.

Fin clips

Fin clips were performed on adult heterozygote zebrafish to extract large quantities of DNA to analyze through quantitative-PCR (qPCR). Portions of the anal fin were clipped and placed in an eppendorf tube containing EDPM and proteinase K. The reaction was allowed to go for 4 hours at 55°C with intermittent vortexing. The proteinase K was rendered inactive by placing the tube in a boiling water bath for 15 minutes. DNA was diluted appropriately.

QPCR

Three primer combinations were used to amplify the DNA extracted from fin clips: EGFP, wnt1, and gsc primers. The zebrafish has only one copy of the goosecoid (gsc) gene and this was used as a reference. qPCR reactions were performed with Sybr-green (SYBR Green PCR Master Mix, Applied Biosystems), ΔCt and ΔΔCt values were determined relative to the gsc reference, and values were used to estimate the transposon insertion copy number.

In situs on heterozygote offspring

In situs were performed according to protocol as described (Ramel, Buckles, and Lekven, 2004) with a GFP probe. Proteinase K treatment on 24hpf embryos was performed for 5 minutes.

Imaging GFP fluorescence in transgenics

All pictures were taken of 24hpf embryos on an Olympus BX-61 compound microscope. Embryos were decorinated and placed on a well slide in glycerol. All bright field images were taken at an exposure time of 2 milliseconds. The exposure time for GFP pictures of the T2A6 derivatives was 2 seconds while the exposure time for the T2AL200R150G was 500 milliseconds. Images were saved as TIF files, then figures assembled in Adobe Photoshop.

Plasmid construction for transient sensor assay

The linker and wnt8.2 3'UTR were ligated into the vector CS2P+EGFP in the XbaI site in a similar fashion as in plasmids constructed for making up the transgenic lines. The regions were ligated into the vector in the forward and reverse direction to continue the previously mentioned control. Once these plasmids were sequenced, portions of the linker region were deleted by inverse PCR.

Injections for transient sensor assay

EGPF mRNA with different 3'UTR elements was injected at a concentration of 25ng/µL with mRFP mRNA at a concentration of 15ng/µL as an internal injection control to show that the same concentration of RNA was injected into embryos. Pictures of the GFP and RFP fluorescence were taken at 24hfp.

CHAPTER III

RESULTS AND DISCUSSION

To test our hypothesis that an important mode of regulation occurs post-transcriptionally through the 3' UTRs on the *wnt8* mRNAs, we made chimeric genes that comprise the gene for GFP in combination with the *wnt8* 3'UTR. Stable GFP transgenic lines were produced with these chimeric genes. Two plasmids were used as vectors to test UTR function (Figure 1). pT2A6 contains EGFP driven by the wnt8 promoter, which drives expression in mesendoderm progenitors. The second plasmid, pT2AL200R150G, contains the EF1-α promoter, which drives broad GFP expression (Urasaki, et al, 2006).

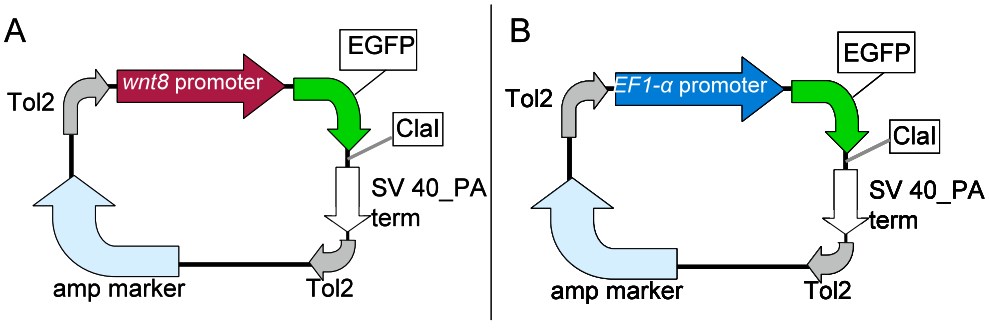

Figure 1: Schematic diagrams of plasmid vectors used in this study. A) pT2A6. B) pT2AL200R150G. The two plasmids differ in the promoter that drives EGFP expression. The *wnt8* promoter comprises 2.7 kb upstream of the *wnt8.1* translation start site (Narayanan and Lekven, unpublished). The EF1-a promoter drives widespread expression.

Using both vectors allowed us to compare the extent of GFP transcription and protein distribution to determine which cells during embryonic development have the ability to regulate wnt8 transcripts via these UTR elements.

Transgenic lines were produced for eight different transgene constructs, four constructs for each vector. For each vector, UTR sequences were ligated into the unique ClaI site between the EGFP coding sequence and the poly-adenylation signal sequence. Thus, elements will be incorporated into reporter mRNAs. We tested the regulatory function of two UTR elements: the linker, located at the 3' end of the *wnt8*.1 coding sequence, and the *wnt8*.2 3' UTR, located at the 3' end of the *wnt8* transcription unit (Figure 2A). Each UTR element was ligated into the ClaI site in both the forward and reverse complement orientations (example for pT2A6 derivatives shown in Figure 2B). Transgenes with UTR elements ligated in the reverse orientation serve as controls for sequence dependence of UTR function. We predicted that if the 3'UTRs negatively regulate gene expression through a sequence dependent manner, transgenes with the reverse complement sequence would express a higher level of GFP protein and/or transcripts. The eight transgenes comprise two vectors with two UTR elements tested in both forward and reverse orientations.

Transgene constructs were coinjected with mRNA encoding the Tol2 transposase into one-cell stage wild-type zebrafish embryos. Injected embryos were raised to adulthood, then outcrossed to wild-type fish. Offspring were scored for EGFP fluorescence at 24

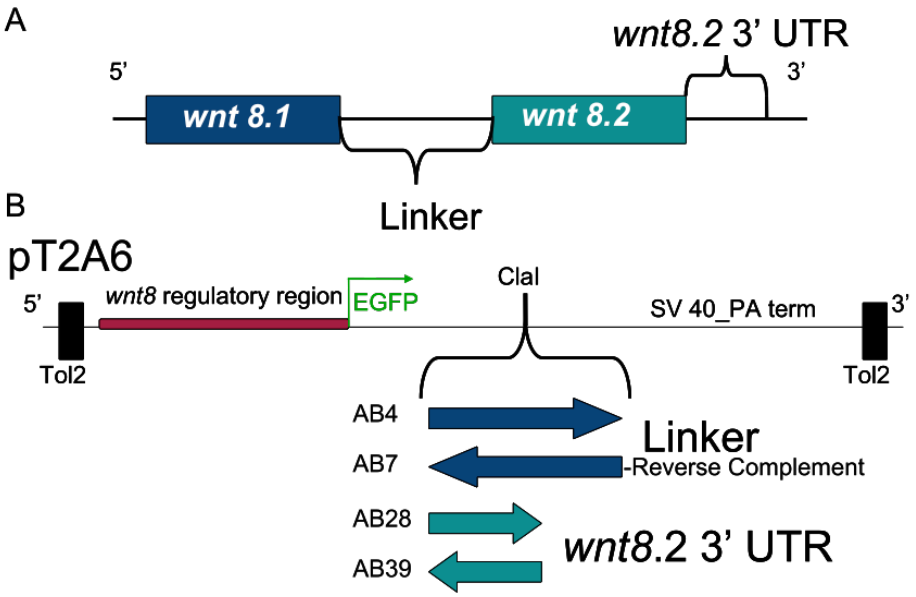

Figure 2. UTR elements in the *wnt8* locus. A) *wnt8* mRNA structure. Boxes represent protein coding regions and lines represent noncoding regions. *wnt8.1* and *wnt8.2* are separated by a UTR called the *wnt8* linker. Another UTR called the wnt8.2 3'UTR is at the 3' end of the *wnt8.2* protein coding region. B) Illustration of the design of reporter transgenes. UTR elements were ligated into the unique ClaI site in pT2A6 (shown) and pT2AL200R150G (not shown). UTR elements are indicated by large arrows. Arrow color correlates UTR element: dark blue indicates the *wnt8* linker, teal indicates the *wnt8.2* 3'UTR. Direction of the arrow indicates the orientation of the insert. The arrows pointing to the right represent the endogenous (wild-type) orientation. Arrows to the left represent the inserts ligated in the reverse orientation. The same ligations were performed into pT2AL200R150G. AB4, AB7, AB28 and AB39 refer to the stable transgenic lines that bear the corresponding transgene; i.e. AB4 is the line that has EGFP with the *wnt8* linker UTR in the wild-type orientation driven by the *wnt8* promoter.

hours post fertilization, and positive embryos (heterozygous F1) were raised to adulthood. Because Tol2 transposition frequently results in multiple insertions in a founder fish, once stable transgenic lines were obtained for all 8 constructs, QPCR was performed to determine the number of insertions in each heterozygote's genome (Table 1). When possible, subsequent comparisons of reporter expression between embryos of

Table 1. The number of insertions for each heterozygote is given.

	Transgenic Line	Description	Number of Insersions
pT2A6 (*wnt8* promoter)			
	AB4	Linker Fwd	3
	AB7	Linker Rev	4
	AB28	wnt8.2 3UTR Fwd	4
	AB39	wnt8.2 3'UTR Rev	4
pT2AL200R150G (EF1-α promoter			
	AB19	Linker Fwd	2
	AB21	Linker Rev	6
	AB47	wnt8.2 3UTR Fwd	4
	AB29	wnt8.2 3'UTR Rev	4

different transgenic lines utilized outcrosses of heterozygotes with similar numbers of transgene insertions.

To characterize reporter expression, I performed in situ hybridizations on embryos at 80% epiboly and 24 hpf, and imaged GFP fluorescence in 24 hpf live embryos. In situ hybridizations on 80% epiboly embryos transgenic for pT2A6 derivatives revealed various classes of expression patterns that could be grouped according to expression level (Figure 3). Endogenous *wnt8* transcripts are expressed at the ventrolateral margin during epiboly, a pattern recapitulated by the AB4 transgene, which comprises the *wnt8*

promoter driving EGFP with the *wnt8* linker as a 3' UTR element (Figure 3A-C; Lekven et al., 2001). Notably, reporter transcripts are not found more than two to three cell diameters away from the margin, which matches the pattern observed for endogenous wnt8. In contrast, transcripts are observed a considerable distance from the margin in embryos of the the AB7 line, which has the *wnt8* linker in the reverse direction (Figure 3D-F). Because the transgenic parents from which the embryos were collected carried multiple transgene insertions, the different expression levels likely correspond to different numbers of transgenes carried by each embryo. However, the difference in staining intensity between AB4 line embryos and AB7 line embryos is consistent between expression classes (for example, compare Fig. 3D to A, 3E to B). These results demonstrate that the *wnt8* linker affects transcript abundance in gastrula stage embryos in a sequence-dependent fashion.

Similarly, the *wnt8*.2 3'UTR regulates transcript abundance during gastrulation, but does not appear to exert as great an effect as the *wnt8* linker (Figure 3G-J). The 3'UTRs in the forward orientation have a smaller abundance of transcripts as compared to the reverse complement control (compare Fig. 3J to G). Thus, the *wnt8*.2 3'UTR also affects transcript abundance in a sequence dependent manner during gastrula stages. A much greater difference can be observed in reporter expression levels in pT2A6-derivative transgenes at 24hfp (Figure 4). In situ hybridizations show that at this stage transcripts are only found at the very tip of the tail, similar to endogenous *wnt8* expression. However, the staining is more intense in the AB7 and AB39 lines, i.e. those lines in which the UTR element is in the reverse orientation, than in the AB4 and AB28

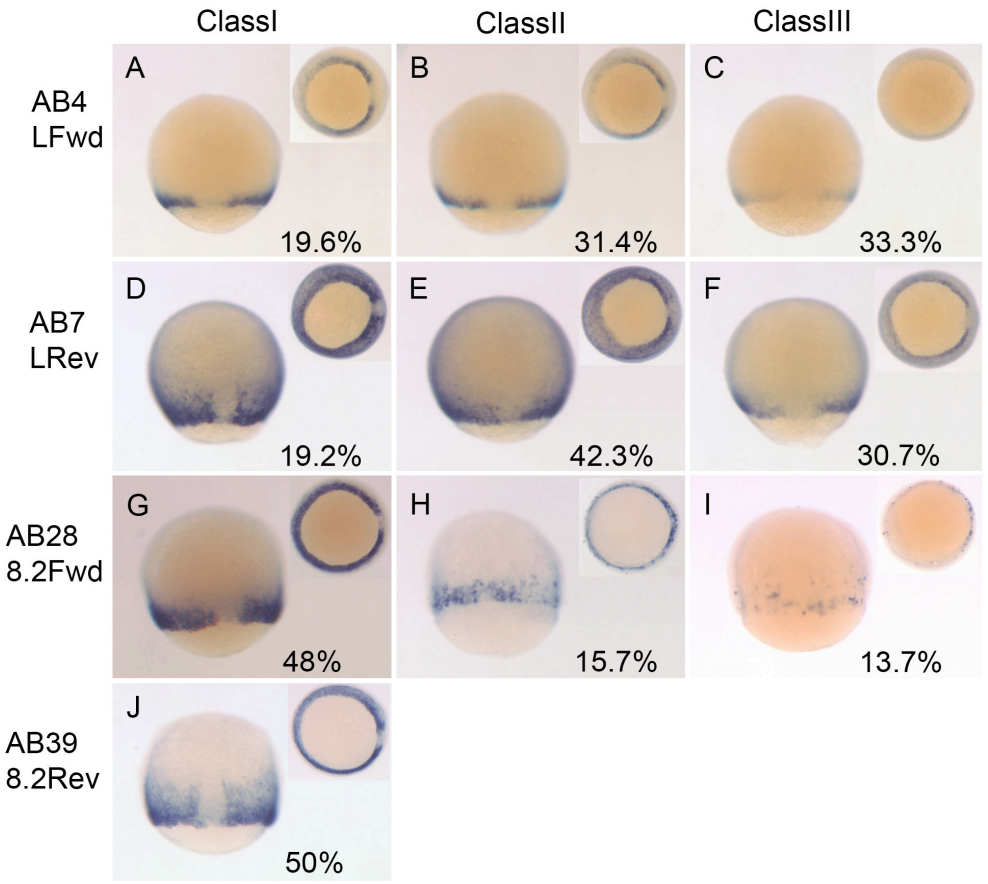

Figure 3. In situ hybridizations to detect EGFP in pT2A6-derivative transgenic embryos at 80% epiboly. Due to the multiple insertions in each line, different degrees of staining were observed in one clutch of embryos. Rows represent each transgenic line with the description to the left. Columns describe the degree of staining observed, class I representing the most staining and class III the least.

lines. Similarly, EGFP fluorescence, which reflects reporter translation, shows a congruent pattern. For example, EGFP fluorescence is broadly distributed in embryos of the AB39 line (Fig. 4L), but is less intense in the AB28 line (Fig. 4I). EGFP has a long

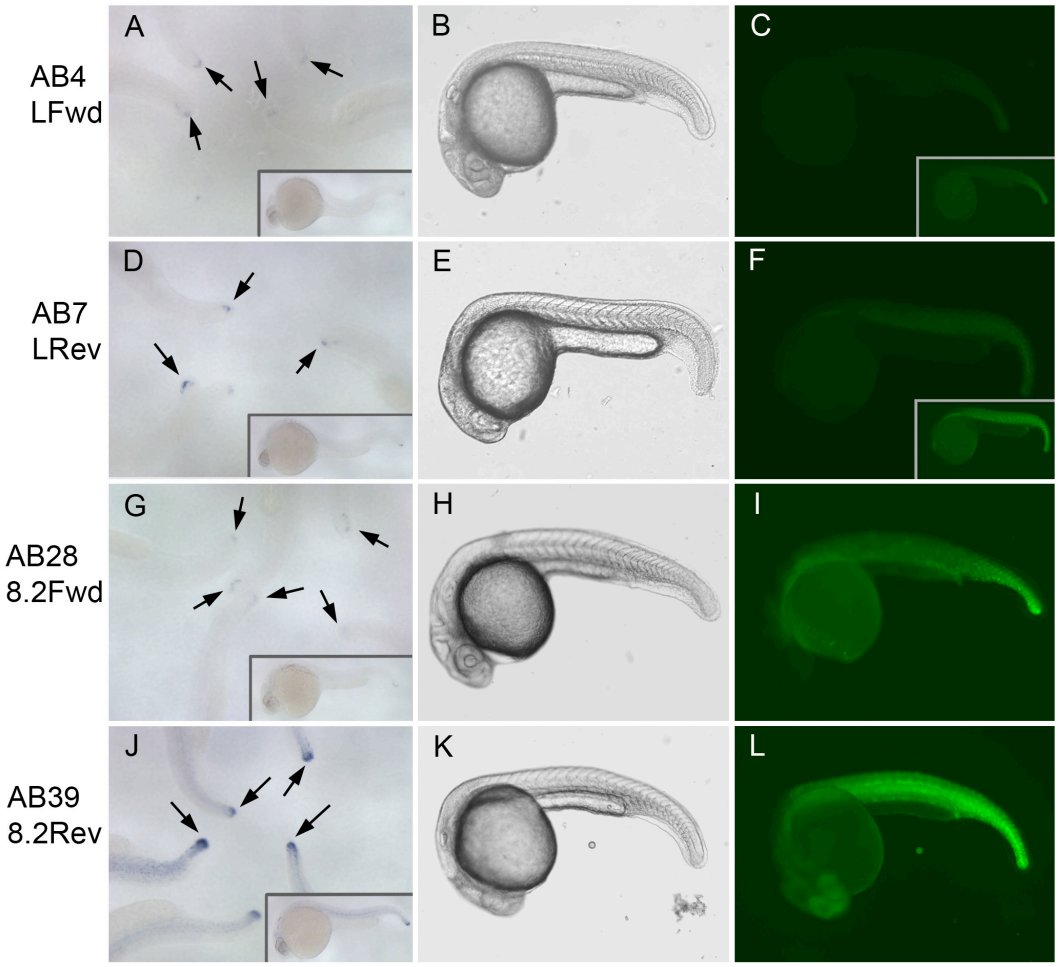

Figure 4. 24 hfp embryos of T2A6 derivative lines. Transgenic line is indicated to the left of each row. (A,D,G,J) In situ hybridizations with an EGFP probe. Arrows point to the tip of the tail where transcripts are observed. The insets show the entire embryo to indicate that there is no other staining. (B,E,H,K) Bright field images of embryos (C,F,I,L) EGFP fluorescence. to show that the embryos look wild type. All fluorescence images taken with the same exposure time. Insets for C and F show EGFP fluorescence at a longer exposure time for AB4 and AB7, respectively.

half life, thus the broader protein distribution compared to the transcription pattern reflects expression at an earlier stage in those mesodermal progenitors. The EGFP

fluorescence in the AB4 and AB7 lines is generally lower than the AB28 and AB39 lines, which may be due to position-dependent effects on the transgenes or to potential transcriptional regulatory roles of the *wnt8* linker since transcriptional regulation through enhancers should not be dependent on the orientation of the enhancer. However, at higher exposure times, a significant difference is observed between the AB4 line that has the linker in the forward direction and the AB7 line that has the linker in the reverse orientation.

A similar pattern is observed with the T2AL200R150G derivatives in that the constructs containing either the *wnt8* linker or the *wnt8*.2 3'UTR in the forward direction have lower reporter expression than those with the UTR elements in the reverse orientation. For example, as detected by in situ hybridization, expression in the AB21 and AB29 lines, that have UTR elements in the reverse orientation, is much greater than in the AB19 and AB47 lines that have the UTR elements in the forward directions (Figure 5). Similarly, EGFP fluorescence observed at 24 hpf reflects these differences in transcriptional levels (Figure 6).

Because the EF1-α promoter is broadly expressed, we may determine whether the UTR-dependent regulatory mechanism is limited to specific tissues. As indicated in Figure 5, transcripts are destabilized in all tissue types that express the EGFP reporter. This means that if transcripts are being destabilized through miRNAs, the miRNAs themselves have to also be distributed throughout the embryo during development. Thus, our results show

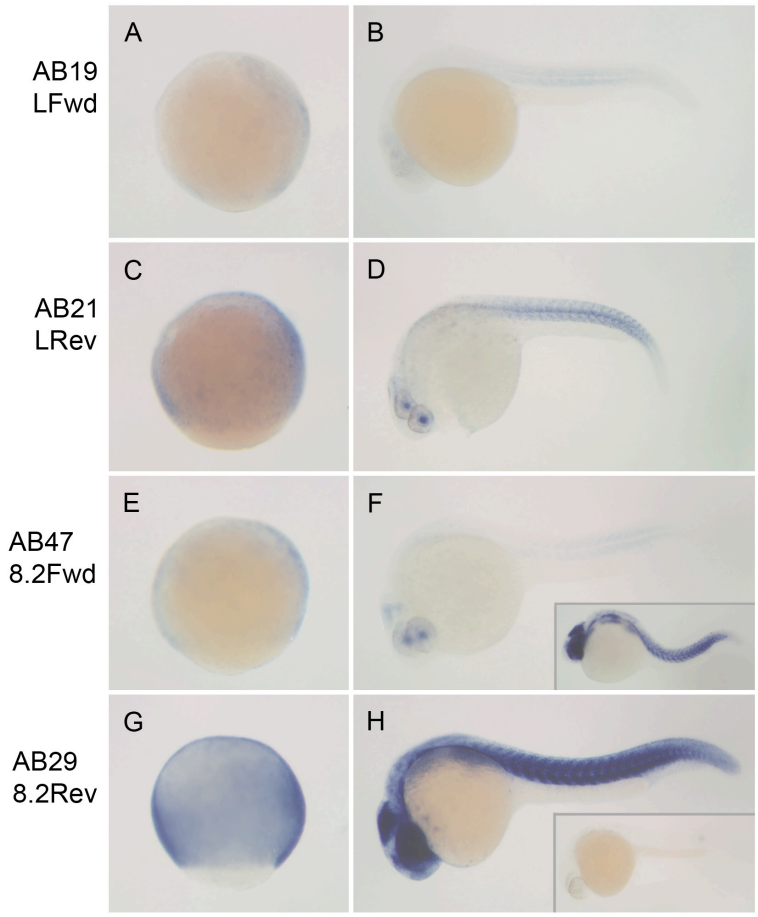

Figure 5. In situ hybridizations to detect EGFP in T2AL200R150G-derivative transgenic lines. (A,C,E,F) 80% epiboly. (B,D,F,H) 24 hpf. The transgenic line, the insert, and its directionality are indicated to the left of each row. The inset in F shows the staining that is observed when the staining reaction is allowed to continue for a longer period of time. The inset in H shows a non-transgenic wild type embryo found in the same clutch indicating that none of the staining observed is background.

that both the *wnt8* linker and the *wnt8*.2 3' UTR reduce transcript levels for their cognate mRNAs in a sequence-dependent manner that is not tissue restricted.

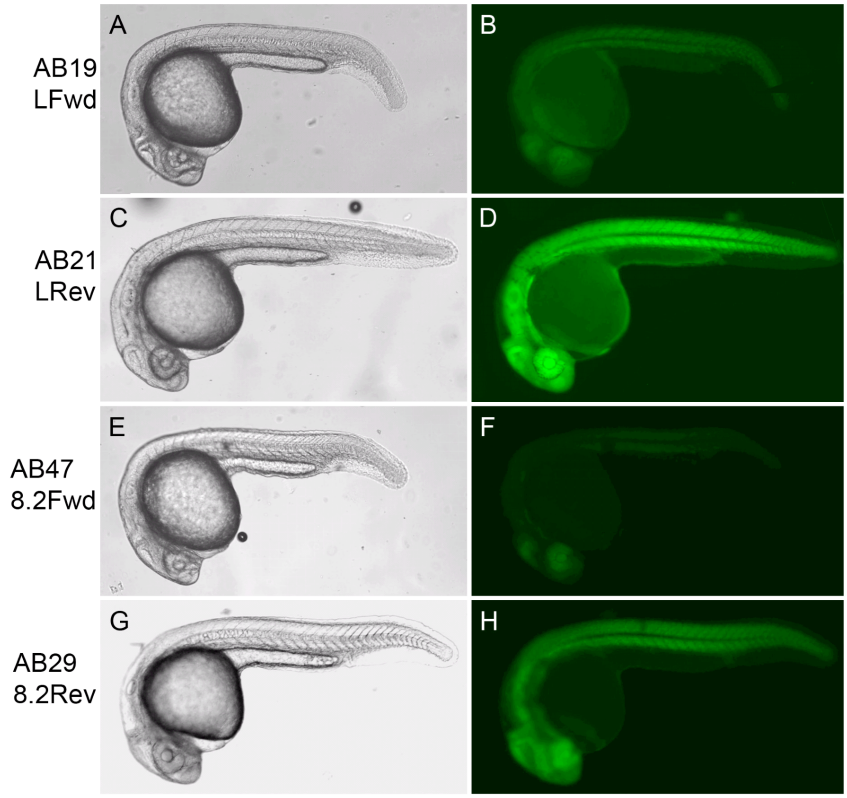

Figure 6. EGFP fluorescence in 24 hpf T2AL200R150G-derivative transgenic embryos. Transgenic line identity is indicated to the left of each row. Left row: bright field image of embryo to the right. Note higher expression level in lines with UTR elements in the reverse orientation. Images in B and D taken with longer exposure than F and H.

To determine the mechanism of *wnt8* UTR-dependent regulation, we used a "transient sensor assay" approach in which synthetic EGFP mRNAs with various portions of the *wnt8* UTRs are injected into one-cell stage embryos and then EGFP fluorescence is

observed at 24 hpf (Figures 7, 8). We limited our focus for this approach to the *wnt8* linker. Synthetic EGFP mRNAs with the *wnt8* linker in the forward orientation are predicted to be negatively regulated by the linker, thus producing lower EGFP fluorescence than synthetic mRNAs with the linker in the reverse orientation. Consequently, if a portion of the UTR is deleted that is essential for regulatory activity, EGFP fluorescence will increase since the transcript cannot be destabilized and more EGFP is produced. Through web-based miRNA target prediction algorithms, several putative miRNA binding sites were identified within the *wnt8* linker region. These were deleted through reverse PCR. Larger portions of the linker were deleted to account for the possibility that other potential miRNA binding sites may have not been identified by the web-based searches (Figure 7). As a control for regulatory activity, several constructs were generated with the 3'UTR in the reverse orientation.

Synthetic EGFP mRNAs with various portions of the *wnt8* linker as 3'UTR elements were coinjected into one-cell stage wild-type embryos, then EGFP fluorescence was observed at 24 hpf (Figure 8). As an internal injection control, synthetic mRNA encoding Red Fluorescent Protein (mRFP) was included to show that the same relative amount of RNA was injected. Consistent with our analysis of the transgenic lines, the *wnt8* linker in the forward orientation has much greater regulatory activity than does its reverse complement sequence. For example, injected EGFP mRNAs with the forward linker UTR produce very little EGFP fluorescence at 24 hpf (LF, Figure 8A-C). In contrast, injected EGFP mRNAs with the reverse linker UTR produce high levels of EGFP fluorescence at 24 hpf (Figure 8D-F).

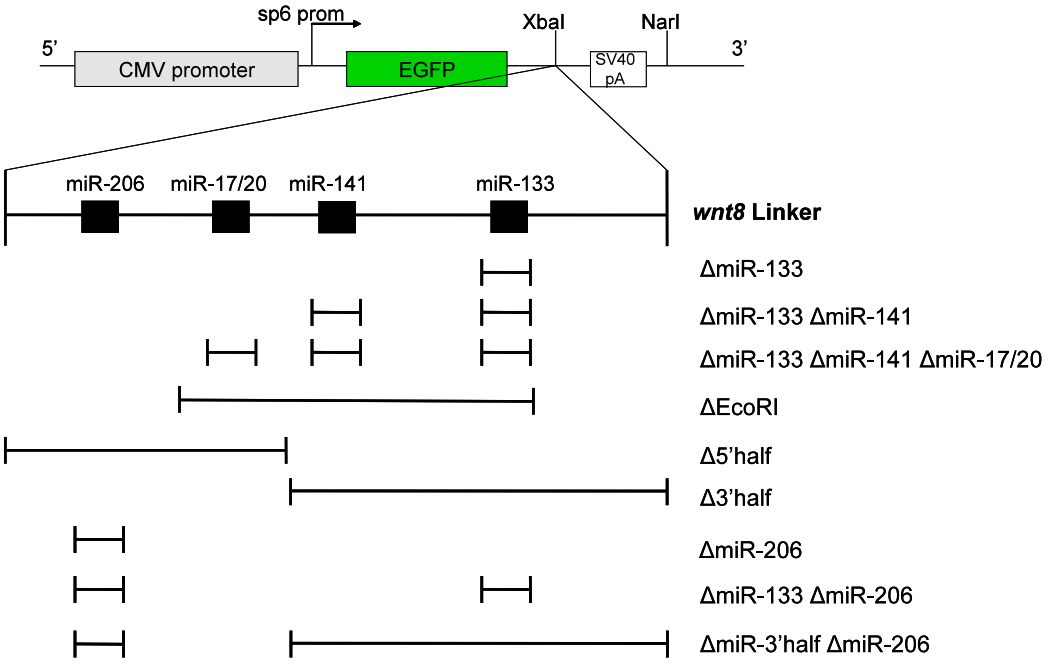

Figure 7. Constructs for the transient sensor assay. The vector is depicted at the top and dotted lines at the XbaI site show where the UTRs were ligated. This is a representative cartoon of the deletions made in the plasmid with the linker in the forward deletions. Lines show the portions of the UTR that were deleted. All deletions were performed similarly with the linker in the reverse direction except for the miR206 sites.

Several deletions within the linker were tested for their effect on regulatory activity. In comparison to the LF construct, the delta5' half construct produced increased EGFP fluorescence relative to the mRFP control (Figure 8G-I). This indicates that the 5' half has an important functional element. In contrast, deleting the 3' half (Figure 8M-O) or the central portion (LF-EcoRI, Figure 8J-L) does not result in more EGFP fluorescence relative to the internal mRFP control than LF. Therefore, the 5' half of the linker possesses a substantial regulatory function. Curiously, deleting the 5' half of the linker

does not recapitulate the degree of EGFP fluorescence as observed by the reverse complement control. This means that the 3' half of the linker still contains activity but

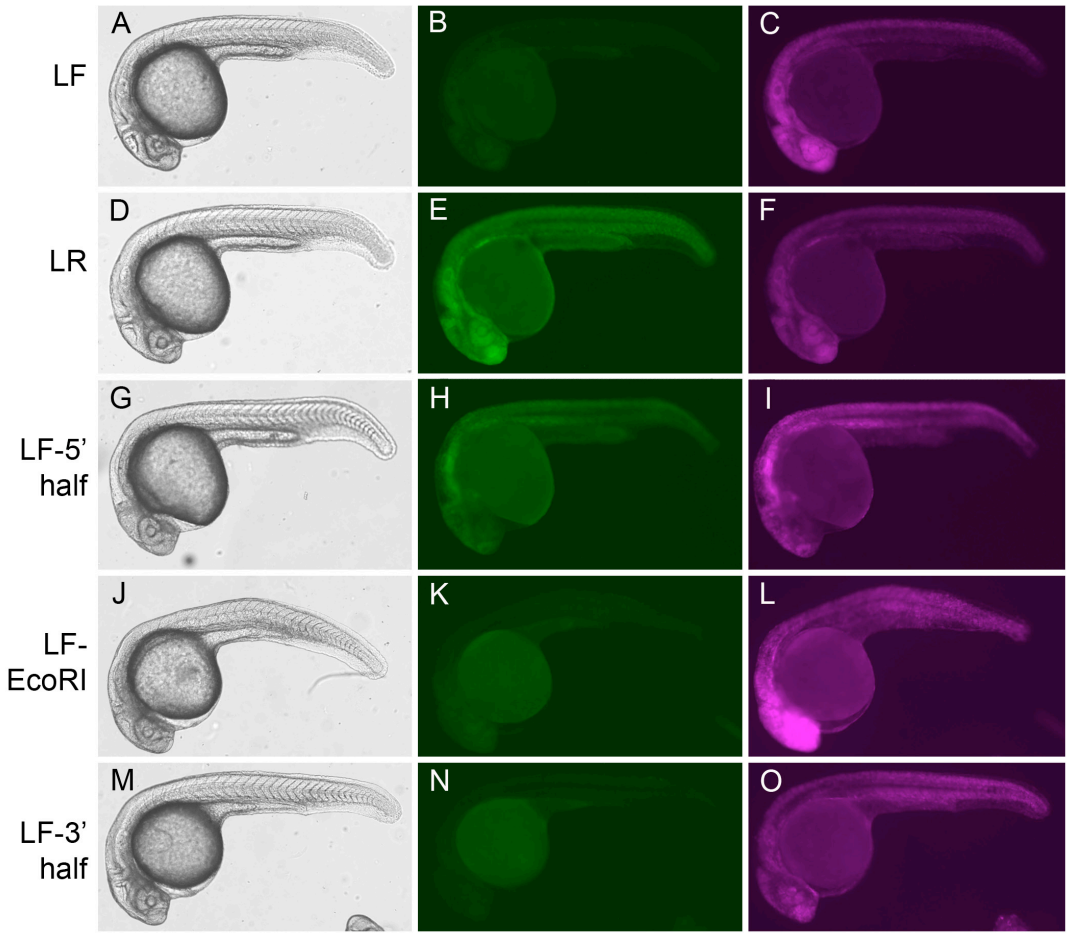

Figure 8. Transient sensor assays identify functional regions in the *wnt8* linker. Images of live 24 hpf embryos. All EGFP images were taken with the same exposure time. mRFP images were taken with the same exposure time. Sensor mRNA injected is indicated to the left of each row. Left column: bright field. Middle column: EGFP fluorescence. Right column: mRFP fluorescence. Each row shows images of the same embryo. mRFP is an injection control. H shows that the 5' portion of the linker region has activity since its deletion prevents the transcript from being destabilized resulting in increased GFP fluorescence.

does not contain the major area of activity. The delta-EcoRI construct result suggests that within the 5' half of the linker, most activity may be attributable to the most 5' 120 bp; however, we cannot exclude the possible synergistic effect of multiple regulatory elements. This possibility is supported by the observation that deletion of individual putative miRNA binding sites within LF did not result in an increase of EGFP fluorescence in transient sensor assays (data not shown).

CHAPTER IV

SUMMARY AND CONCLUSION

Our objective of this study was to determine whether an important mode of regulation occurs post-transcriptionally through the 3' UTRs on the *wnt8* mRNAs and whether this mode of regulation is tissue restricted. By including internal controls we could further determine whether these UTR sequences are critical. For the first part of the project we produced stable transgenic lines comprising the EGFP gene containing alternate UTR sequences. Through these stable transgenic lines we can say with confidence that both the *wnt8* linker and the *wnt8*.2 3'UTR function to destabilize transcripts through a sequence dependent manner. Furthermore, by comparing the T2A6 derivatives to the T2AL200R150G derivatives, we can conclude that the mode of regulation is not tissue restricted.

The second part of the project focused on pinpointing the sequences that were responsible for transcript destabilization within the *wnt8* linker. Various deletions were performed and synthetic EGFP mRNAs were made. A transient sensor assay approach was utilized in which mRNA was injected at the 1 cell stage and GFP fluorescence was assessed at 24hpf. Large deletions of the *wnt8* linker showed that an important functional element is found at the 5' end. Further deletions have been made and will be injected in the same manner to further narrow down this region of interest. Specific putative miRNA binding sites were also assessed for activity but no increase in GFP fluorescence was observed

(data not shown). The same approach is being used with the *wnt8*.2 3'UTR to find the critical sequences which is currently in progress.

Further experiments are in progress in which we are manipulating Wnt8 expression to determine how altering the duration of Wnt8 production affects normal vertebrate embryonic development through these alternate UTRs. Several plasmids have been constructed with the same controls as previously mentioned. These plasmids will be injected do see what phenotypes are observed and how important this mode of regularity is during early vertebrate embryonic development.

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